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Report

The Role of Rapid, Local, Postsynaptic Protein Synthesis in Learning-Related Synaptic Facilitation in *Aplysia*

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Summary

The discovery that dendrites of neurons in the mammalian brain possess the capacity for protein synthesis [1] stimulated interest in the potential role of local, postsynaptic protein synthesis in learning-related synaptic plasticity [2]. But it remains unclear how local, postsynaptic protein synthesis actually mediates learning and memory in mammals. Accordingly, we examined whether learning in an invertebrate, the marine snail *Aplysia*, involves local, postsynaptic protein synthesis. Previously, we showed that the dishabituation and sensitization of the defensive withdrawal reflex in *Aplysia* [3, 4] require elevated postsynaptic Ca^{2+}, postsynaptic exocytosis, and functional upregulation of postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors [5]. Here, we tested whether the synaptic facilitation that underlies dishabituation and sensitization in *Aplysia* [6] requires local, postsynaptic protein synthesis. We found that the facilitatory transmitter, serotonin (5-HT), enhanced the response of the motor neuron to glutamate, the sensory neuron transmitter, and this enhancement depended on rapid protein synthesis. By using individual motor neurites surgically isolated from their cell bodies, we showed that the 5-HT-dependent protein synthesis occurred locally. Finally, by blocking postsynaptic protein synthesis, we disrupted the facilitation of the sensorimotor synapse. By demonstrating its critical role in a synaptic change that underlies learning and memory in a major model invertebrate system, our study suggests that local, postsynaptic protein synthesis is of fundamental importance to the cell biology of learning.

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Results

To examine whether the postsynaptic facilitatory processes that mediate, in part, dishabituation and sensitization in *Aplysia* [5, 7] involve local protein synthesis, we used sensory and motor neurons individually dissociated from the central nervous system (CNS) and placed into cell culture [7–9]. This permitted us to specifically isolate the contribution of local, postsynaptic protein synthesis to synaptic facilitation.

Enhancement of the Glutamate Response in Motor Neurons by 5-HT Depends on Rapid Protein Synthesis

The presynaptic transmitter at the sensorimotor synapse of *Aplysia* appears to be glutamate [10, 11] (but see [12]). Serotonin (5-HT), an endogenous facilitatory transmitter that mediates the dishabituation and sensitization of the withdrawal reflex [13–15], enhances the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated component of the response of isolated LFS [16] siphon motor neurons in cell culture to brief applications (puffs) of glutamate (2 mM in perfusion medium with 0.02% Fast Green). The glutamate was ejected from a micropipette onto the initial segment of the motor neuron's major neurite via a Picospritzer, and the evoked potential was recorded with an intracellular microelectrode (Figure 1A). We first tested whether the 5-HT-dependent enhancement of the evoked glutamate response (Glu-EP) in isolated motor neurons requires protein synthesis. In our initial experiments, we stimulated the motor neuron with glutamate once every 10 s. A 10 min application of 5-HT produced the enhancement of the Glu-EP that persisted for more than 40 min (Figures 1B and 1B′) (see also [7]). By contrast, the enhancement of the Glu-EP produced by a 1 min application of 5-HT was short lived, lasting less than 5 min after 5-HT washout. Furthermore, the persistence of the enhancement of the Glu-EP induced by a 10 min application of 5-HT did not depend on the once per 10 s glutamate stimulation rate. When glutamate was delivered with a much more spaced stimulation protocol (once every 5 min), a 10 min 5-HT treatment also induced the prolonged enhancement of the Glu-EP (Figures 1C and 1C′).

To determine whether the 5-HT-dependent enhancement of the Glu-EP requires protein synthesis, we used the irreversible cell-membrane-permeant protein-synthesis inhibitor emetine. The inhibitor (1–3 μM) was bath applied 20 min before the onset of 5-HT and remained in the bath throughout the experiment. Emetine abolished the 5-HT-dependent enhancement (Figures 2A and 2B) but did not depress the baseline Glu-EP in the motor neuron. This result suggests that 5-HT triggers rapid de novo protein synthesis in the isolated motor neuron. But because the protein-synthesis inhibitor was present for 20 min before the onset of 5-HT, it is possible that the effect of the inhibitor, rather than

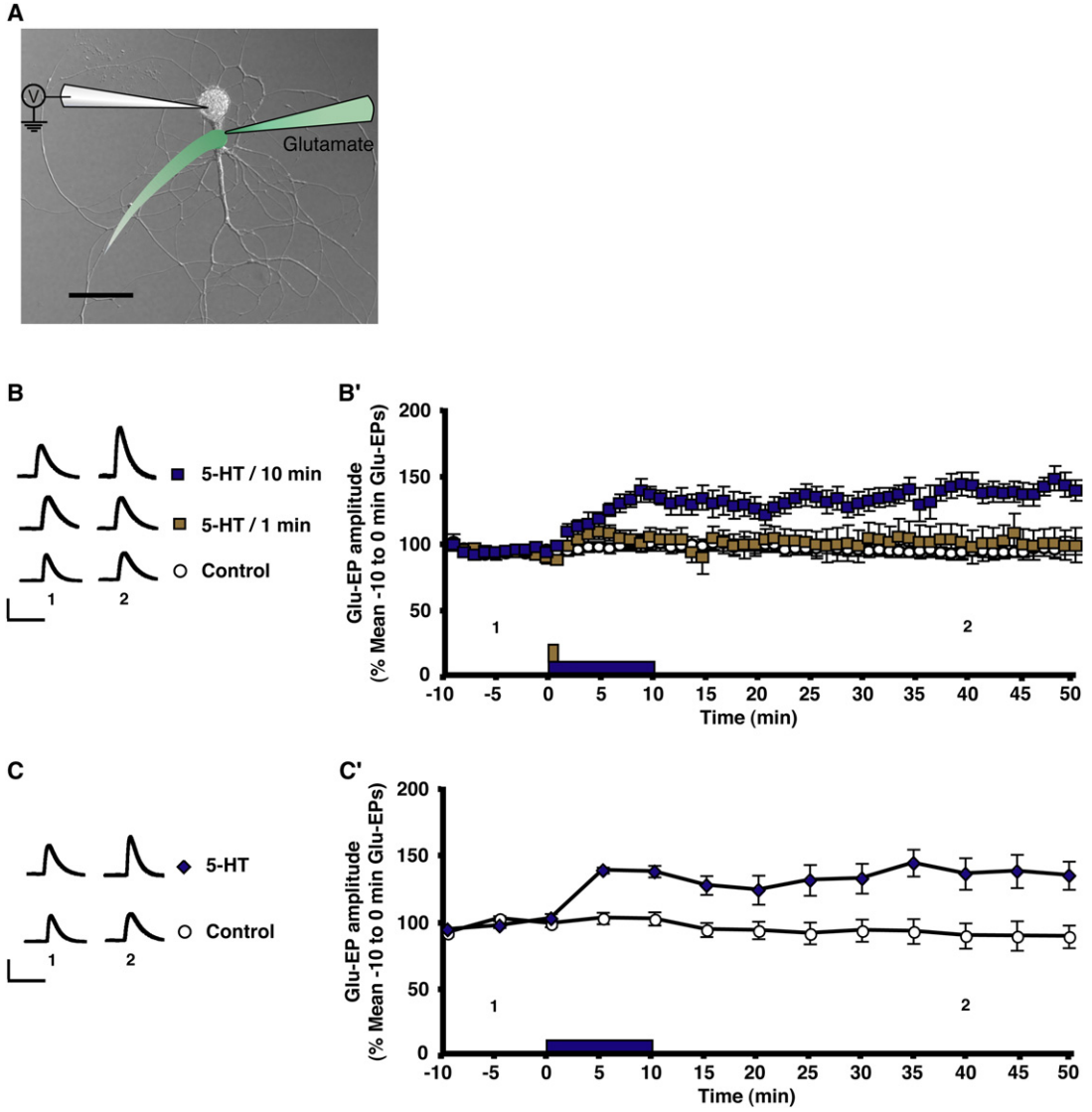

Figure 1. 10 Min but Not 1 Min of 5-HT Stimulation Causes Prolonged Enhancement of the Glutamate-Evoked Response in Motor Neurons

(A) Composite micrograph and cartoon depicting the experimental arrangement. The cell cultures consisted exclusively of isolated small siphon (LFS) motor neurons [16]. Pulses of glutamate were pressure ejected from a micropipette (right) onto the initial segment of the major neurite of the motor neuron once every 10 s (except for the experiment shown in [C]). Fast Green was used for the visualization of the glutamate. The evoked glutamate potentials (Glu-EPs) were recorded from the motor neuron's cell body with a sharp microelectrode (left). See the Experimental Procedures for additional information. The scale bar represents 50 μm.

(B) Sample Glu-EPs. Each pair of traces shows responses from one experiment. Traces marked "1" represent sample Glu-EPs evoked at the 5 min time point from the experiments summarized in (B′); those marked "2" represent Glu-EPs evoked at the 40 min time point. Scale bars represent 10mV and 500 ms.

(B′) Comparison of the effects of 10 min (blue bar) and 1 min (brown bar) of 5-HT stimulation. Each symbol in the graph represents the mean normalized amplitude of six consecutive Glu-EPs. Motor neurons received 10 min of 5-HT (n = 9), 1 min of 5-HT (n = 10), or perfusion medium alone (n = 10; control group). The Glu-EP values were log transformed so that a parametric one-way ANOVA could be performed on the data for the 40 min time point. The ANOVA indicated that the group differences were highly significant ($F_{[2,26]} = 7.77$, $p < 0.003$). The mean Glu-EP in the 10 min 5-HT treatment group at the 40 min time point (147 ± 8%) was significantly larger than that at the same time point in the 1 min 5-HT treatment group (110 ± 11%), as well as in the group that received the control treatment (100 ± 4%; post-hoc tests, $p < 0.01$ for each comparison). The difference between the 1 min 5-HT and the control groups was not significant ($p > 0.05$). The numbers below the data indicate the times at which the sample Glu-EPs shown in (B) were recorded. Error bars represent ± SEM.

(C) Sample Glu-EPs from experiments in which glutamate was applied to the motor neuron once every 5 min. Otherwise, the procedures were identical to those used in the experiments in which the motor neuron was stimulated with glutamate every 10 s. See the legends for (A) and (B) for additional details. Scale bars represent 10mV and 500 ms.

(C′) Effect of 5-HT when the motor neuron was stimulated with glutamate at a low rate. Each symbol represents the mean normalized amplitude of six Glu-EPs. Values were normalized to the Glu-EP recorded immediately before 5-HT application (t = 0 min). Either 5-HT (n = 7) or normal perfusion medium (n = 6, control group) was applied for 10 min. The Glu-EP in the 5-HT-treated group at the 40 min time point (140 ± 10%) was significantly larger than was the control Glu-EP at 40 min (88 ± 9%; $p < 0.004$, unpaired t test). The numbers below the data indicate the times at which the sample Glu-EPs shown in (C) were recorded. Error bars represent ± SEM.

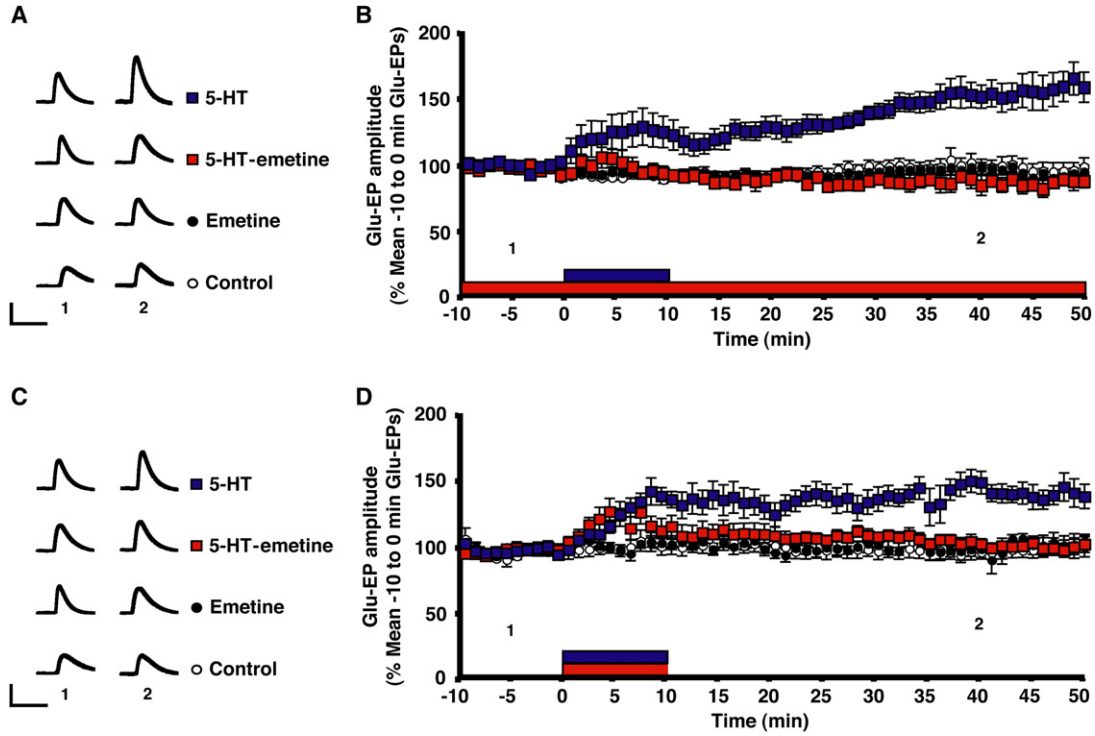

Figure 2. Rapid Protein Synthesis Is Required for Enhancement of the Glutamate Response in Motor Neurons

(A) Sample Glu-EPs recorded at the 5 min ("1") and 40 min ("2") time points in one experiment from each group. The motor neuron was stimulated with glutamate once every 10 s. See the legends of Figures 1A and 1B for details. Scale bars represent 10mV and 500 ms.

(B) Effect of starting emetine treatment prior to 5-HT treatment. Neurons received 5-HT (blue bar, n = 6), 5-HT in the presence of emetine (red bar, n = 6), emetine alone (n = 6), or neither 5-HT nor emetine (n = 6, control group). The group differences for the 40 min time point were significant ($F_{[3,20]} = 20.41$, $p < 0.0001$). The mean Glu-EP in the 5-HT group (152 ± 9%) was greater than that in the 5-HT-emetine (87 ± 6%), the emetine alone (98 ± 4%), and the control (99 ± 6%) groups ($p < 0.001$ for each comparison). There were no significant differences among the 5-HT-emetine, emetine alone, and control groups at 40 min ($p > 0.05$). The numbers below the data indicate the times at which the sample Glu-EPs shown in (A) were recorded. Error bars represent ± SEM.

(C) Sample Glu-EPs for the experiments shown in (D). Refer to (A).

(D) Effect of simultaneous 5-HT and emetine treatment. Refer to (B). We wished to perform one-way ANOVAs on each time point in this experiment. Therefore, we first performed a two-way ANOVA on the overall data. The two-way ANOVA indicated that the differences among the experimental groups were highly significant ($F_{[3,23]} = 11.61$, $p < 0.0001$). There was also a significant interaction between experimental treatment and time ($F_{[3,23]} = 3.44$, $p < 0.0001$). The overall mean Glu-EP for the 5-HT group (131 ± 1%, n = 8) was significantly greater than that in the 5-HT-emetine (109 ± 1%, n = 7), the emetine alone (102 ± 1%, n = 6), and control (101 ± 1%, n = 6) groups ($p < 0.05$ for each comparison). Furthermore, the overall mean Glu-EP in the 5-HT-emetine group was significantly greater than that in both the emetine alone and control groups ($p < 0.05$ for each comparison). Overall, the difference between the means in the emetine alone and control groups was not significant ($p > 0.05$). A one-way ANOVA on the 40 min data indicated that the group differences were highly significant ($F_{[3,23]} = 14.33$, $p < 0.0001$). Post-hoc tests indicated that the Glu-EP in the 5-HT group (147 ± 8%) at this time was significantly greater than that in the 5-HT-emetine group (105 ± 4%), as well as the emetine group (100 ± 6%) and control group (99 ± 6%; $p < 0.0001$ for each comparison). The differences among the latter three groups were not significant at 40 min ($p > 0.05$). To determine the earliest time at which the inhibition of protein synthesis disrupted 5-HT-dependent enhancement of the Glu-EP, we performed one-way ANOVAs on the data for the other time points. The time at which the difference between the amplitude of the Glu-EP in the 5-HT group and that in the 5-HT-emetine group first became significant was t = 13 min (136 ± 9% versus 110 ± 9%, $p < 0.05$). Error bars represent ± SEM.

being due to the disruption of de novo protein synthesis, was due instead to the elimination of proteins whose presence is required in the cell for facilitation. The elimination of such essential proteins by emetine might occur prior to the onset of 5-HT if the proteins turn over rapidly. To test this possibility, we performed experiments in which emetine and 5-HT were added coincidentally. In these experiments, emetine also blocked the 5-HT-dependent enhancement of the Glu-EP, although the disruptive effect of the protein-synthesis inhibitor was delayed (Figures 2C and 2D). One-way analyses of variance (ANOVAs) on the data for each of the time points revealed that the difference between the 5-HT group and 5-HT-emetine group first became significant at t = 13 min (see Figure 2D for details). These results suggest that the enhancement of the Glu-EP depends on protein synthesis within 3 min after the washout of 5-HT.

To test whether emetine's blockade of the enhancement of the glutamate response was due to a nonspecific action of the drug, we also examined the effect of a cell-membrane-permeant, but reversible, inhibitor of protein synthesis, cycloheximide. In these experiments, the drug (50 μM) was washed into the culture dish 10 min before the start of testing and then washed out with the 5-HT. Cycloheximide also blocked 5-HT's enhancement of the Glu-EP (Figure S1 in the Supplemental Data available online).

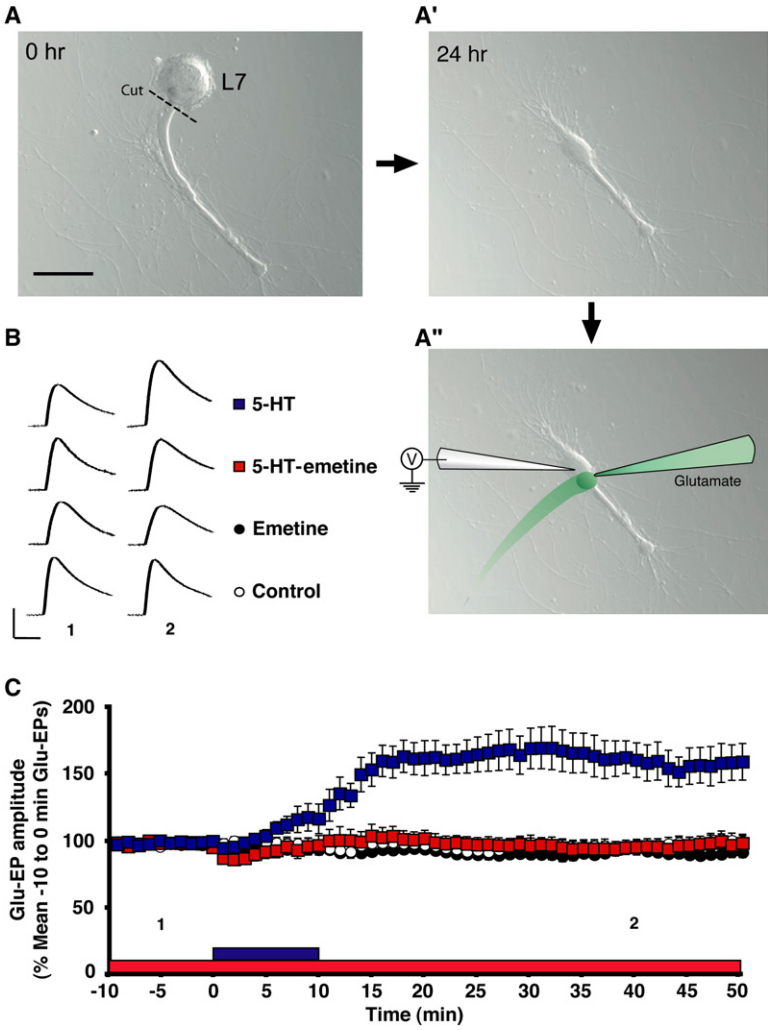

Figure 3. Emetine Blocks Enhancement of the Glutamate-Evoked Response in a Surgically Isolated Motor Neurite

(A) Gill motor neuron L7 isolated in cell culture prior to soma removal. The dashed line shows where the main neurite was severed. The scale bar represents 100 μm.

(A′) The neurite was allowed to recover 24–48 hr after it was severed. Note the swelling of the neurite proximal to the former site of the cell body.

(A″) The neurite was impaled with a sharp electrode. The stimulation and recording methods were like those used in the experiments that used the LFS motor neurons with their cell bodies (Figures 1 and 2). Glutamate was applied to the isolated neurite every 10 s. See Figure 1B and the Experimental Procedures for recording and stimulation methods.

(B) Sample Glu-EPs recorded at the 5 min ("1") and 40 min ("2") time points in one experiment from each group. The scale bars represent 10mV and 500 ms.

(C) Effect of the inhibition of protein synthesis on the enhancement of the glutamate response in the neurite. The Glu-EP values were log transformed, and one-way ANOVA was performed on data for the 40 min time point. The differences among the four groups at this time were highly significant ($F_{[3,25]}$ = 15.45, $p < 0.0001$). The mean Glu-EP in the 5-HT group (159 ± 13%, n = 8) was significantly greater than that in the 5-HT-emetine (98 ± 5%, n = 7), as well as in the emetine alone (95 ± 2%, n = 7) and control (99 ± 5%, n = 7) groups ($p < 0.001$ for each comparison). There were no other significant differences among the groups. The numbers below the data indicate the times at which the sample Glu-EPs shown in (B) were recorded. Error bars represent ± SEM.

Enhancement of the Glutamate Response in the Surgically Isolated Motor Neurite Also Depends on Protein Synthesis

The speed with which the inhibition of protein synthesis disrupted the 5-HT-dependent enhancement of the motor neuron's response to glutamate is consistent with the idea that 5-HT causes the rapid synthesis of proteins within the processes of the motor neuron rather than in the cell body. To further examine this idea, we performed experiments on isolated neurites of the identified giant motor neuron L7, which innervates the gill and mantle shelf [17]. The L7 cell was dissociated from the abdominal ganglion and placed into cell culture alone (Figure 3A). After 24 hr, the main neurite of the L7 cell was transected and the cell body was removed (Figures 3A and 3A′). (Note that neurites of invertebrate neurons can survive the loss of their cell bodies and continue to function normally for days to weeks [18, 19].) Twenty-four to forty-eight hours after isolating the neurite of the L7 cell from its cell body, we impaled the neurite with a micropipette and stimulated it with glutamate (Figure 3A″). As was the case for the isolated siphon motor neuron (Figure 1), a 10 min application of 5-HT produced the prolonged enhancement of the Glu-EP, which was blocked with emetine (2.5 μM) present in the culture dish (Figures 3B and 3C). Therefore, the motor neurite by itself can support the persistent enhancement of the Glu-EP; moreover, the enhancement depends on local protein synthesis.

Synaptic Facilitation Requires Postsynaptic Protein Synthesis

We next asked whether local, postsynaptic protein synthesis plays a role in the synaptic facilitation that mediates the dishabituation and sensitization of the withdrawal reflex. To address this question, we used cultured synapses between a single sensory neuron and a single LFS siphon motor neuron [5, 20] (Figure 4A). The sensory neuron and motor neuron were each impaled with microelectrodes. The sensory neuron was stimulated every 5 min, and the excitatory postsynaptic potential (EPSP) recorded in the motor neuron. In some experiments, 5-HT was added to the perfusion medium after the second test stimulus. In other experiments, no 5-HT was added; we included these experiments ("test alone") to control for the significant homosynaptic depression exhibited by the sensorimotor synapse in response to the low-frequency stimulation of the sensory neuron [21]. As reported recently [5], the 5-HT treatment produced facilitation of the EPSP that lasted for at least 50 min after the washout of the drug. We examined whether this prolonged facilitation requires postsynaptic

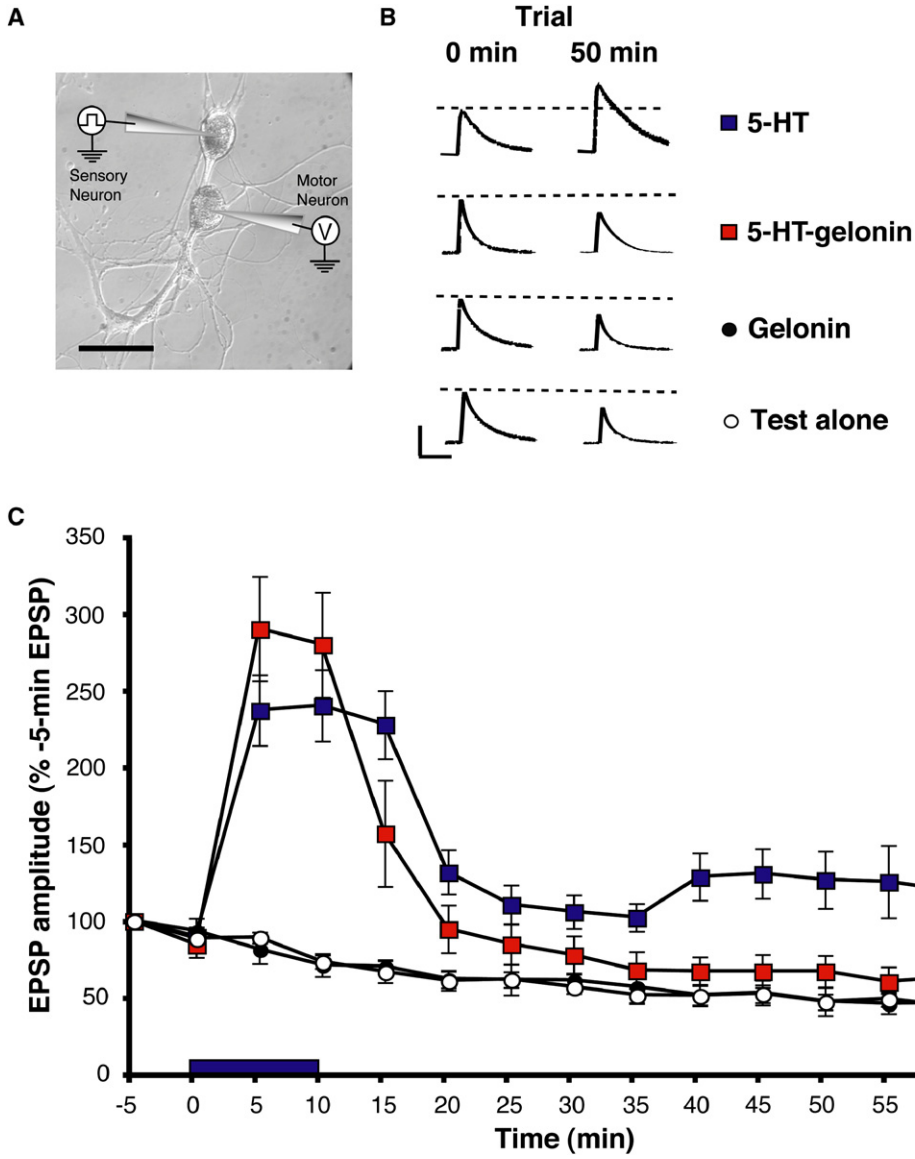

Figure 4. Postsynaptic Inhibition of Protein Synthesis Blocks Persistent Synaptic Facilitation

(A) Experimental arrangement. The scale bar represents 50 μm.

(B) Representative EPSPs. Trial times correspond to those shown in the graph in (C). Scale bars represent 10mV and 40 ms.

(C) Mean normalized amplitude of the EPSPs. The EPSP values were log transformed prior to the performance of a two-way ANOVA on the overall data. The two-way ANOVA indicated that the differences among the experimental groups were significant ($F_{[3,22]} = 24.76$, $p < 0.0001$). There was also a significant interaction between experimental treatment and time ($F_{[3,22]} = 11.22$, $p < 0.0001$). SNK post-hoc tests showed that the overall mean EPSP in the 5-HT group (144 ± 7%, n = 7) was significantly greater than that in the 5-HT-gelonin (112 ± 10%, n = 6), the gelonin alone (62 ± 2%), and the test alone (61 ± 2%, n = 6) groups ($p < 0.05$ for each comparison). There was also a significant difference between the 5-HT-gelonin and the gelonin alone groups, and between the 5-HT-gelonin and the test alone groups ($p < 0.05$ for each comparison). There was no significant overall difference between the gelonin alone and test alone groups ($p > 0.05$). One-way ANOVAs were performed on every time point for the experiment. These revealed that the difference between the 5-HT and 5-HT-gelonin EPSPs became significant at t = 20 (132 ± 15% versus 95 ± 16%, $p < 0.05$). Moreover, the differences between the 5-HT-gelonin EPSP and gelonin alone EPSP (61 ± 6%), as well as between 5-HT-gelonin EPSP and the test alone EPSP (62 ± 4%), were not significant at t = 20 ($p > 0.05$ for each comparison). The numbers below the data indicate the times at which the sample EPSPs in (B) were recorded. Error bars represent ± SEM.

protein synthesis by injecting the cell-membrane-impermeant inhibitor gelonin [22, 23] into the motor neuron prior to testing. Postsynaptic gelonin significantly reduced the facilitation after the washout of 5-HT. Interestingly, the protein-synthesis inhibitor did not affect facilitation while the drug was present in the bath, consistent with the idea that short-term facilitation does not require postsynaptic protein synthesis [24–26] (but see below).

To determine the earliest time at which the postsynaptic gelonin adversely affected facilitation, we performed a one-way ANOVA on each test point in the experiment. The results indicated that the difference between the 5-HT and 5-HT-emetine groups was significant by the 20 min test (see Figure 4 for details). Furthermore, the 20 min test was also the earliest point at which the differences between the 5-HT-emetine and the emetine

alone groups, as well as between the 5-HT-emetine and test alone groups, were insignificant. Therefore, the inhibition of protein synthesis impaired synaptic facilitation by 10 min after 5-HT washout.

Discussion

Clusters of ribosomes have been found in the postsynaptic region of the giant synapse of a cephalopod mollusk, the squid [27]; this finding indicates that local, postsynaptic translation can occur in at least some invertebrate synapses and provides additional support for the present results. Our data agree with those from mammalian studies pointing to a critical role for local, postsynaptic protein synthesis in learning-related synaptic plasticity, including long-term potentiation (LTP) and long-term depression (LTD) (reviewed in [28]), as well as in certain forms of learning [29]. Particularly intriguing in light of our results are recent data showing that 15 min of stimulation with the monamine dopamine triggers the synthesis of the GluR1 subunit of AMPA receptors in dendrites of hippocampal neurons in culture [30]. We have found that 5-HT produces the upregulation of the functional expression of AMPA receptors in *Aplysia* motor neurons [5, 7]. The idea that 5-HT treatment triggers the rapid synthesis of AMPA receptors in motor neurites is therefore attractive [31]. At present, however, we do not know the identity of the local postsynaptic protein(s) whose synthesis is stimulated by 5-HT.

The persistent in vitro synaptic facilitation produced in the present study by 10 min of 5-HT treatment (Figure 4) might represent a type of intermediate-term facilitation (ITF), which has been previously described for the sensorimotor synapse [24, 26]. This form of synaptic plasticity lasts more than 30 min, but less than 3 hr, and requires protein synthesis but not gene transcription [24, 25]. A role for postsynaptic protein synthesis in ITF has not been shown previously. Our results suggest that at least some forms of ITF involve local protein synthesis in the motor neuron.

The rapidity of the requirement for postsynaptic protein synthesis in the enhancement of the glutamate response (Figures 2 and 3) and in synaptic facilitation (Figure 4) in the present study is unprecedented in studies of learning-related plasticity in *Aplysia*. Our results indicate that the onset of de novo local protein synthesis recruited in the motor neuron by a 10 min application of 5-HT is not later than 10 min after the washout of the drug. Because this estimate includes the time required for the protein synthesis inhibitor to cross the cell membrane (in the case of the bath-applied inhibitors) and reach critical sites for protein synthesis within the neurites of the motor neuron, 5-HT might actually trigger de novo synthesis of proteins in motor neurites significantly earlier. Previous results indicate that anisomycin, a cell-membrane-permeant inhibitor of protein synthesis, can produce ~90% inhibition of leucine incorporation in the abdominal ganglion of *Aplysia* within 15 min [32]. Furthermore, the injection of emetine into intact *Aplysia* inhibits protein synthesis, as measured by leucine incorporation, within central ganglia by more than 90% within 30 min [33]. These results provide support for the present study by indicating that inhibitors of protein synthesis can significantly reduce amino acid incorporation in the CNS of *Aplysia* within minutes. Although unprecedented in studies of *Aplysia*, a requirement for rapid protein synthesis has been documented in studies of synaptic plasticity in the mammalian brain (e.g., [34–36]).

Short-term facilitation (STF) in *Aplysia* is generally thought to last for less than 30 min and not to require protein synthesis [37–40] (but see [24]). The present results challenge this common view. We observed that, although the inhibition of postsynaptic protein synthesis did not affect synaptic facilitation while 5-HT was present in the bath, it quickly (within 10 min) impaired facilitation after the washout of 5-HT (Figure 4C). Interestingly, Ghirardi et al. [24] found that treatment with five spaced pulses of very low concentrations of 5-HT (1–10 nM, compared to the 10–20 μM concentration used in the present experiments) produced the facilitation of sensorimotor synapses in culture that persisted for 0.5 hr after the 5-HT treatment and did not depend on protein synthesis. Because the 5-HT treatment period lasted 1.5 hr, this means that very low doses of 5-HT can yield persistent facilitation in the absence of protein synthesis. Because of the lack of the persistent facilitation's dependence on protein synthesis, Ghirardi et al. referred to it as STF rather than ITF, despite its duration. If a requirement for protein synthesis and the lack of a requirement for gene transcription are considered to be the defining properties of ITF, then our results indicate that 10 min of 10–20 μM 5-HT can produce ITF whose onset is within 10 min after washout of 5-HT.

In summary, this study, together with our earlier study [5], presents compelling evidence that the dishabituation and sensitization of the withdrawal reflex in *Aplysia* depend on rapid, local synthesis of proteins in the postsynaptic motor neuron. Our data therefore provide strong support for the idea that local, postsynaptic protein synthesis is an important general mechanism in learning and memory.

Experimental Procedures

Cell Cultures

Small siphon (LFS) [16] motor neurons were individually dissociated from the abdominal ganglia of adult *Aplysia californica* (80–120 g) and isolated in cell culture [7]. The cell cultures used in the glutamate puff experiments consisted exclusively of isolated motor neurons or motor neurites. Experiments were performed on the neurons 3–5 days after they were placed into culture. In experiments on isolated motor neurites, the large identified motor neuron, L7, was dissociated from juvenile animals (1–4 g) and placed into cell culture. After 24 hr, the cell body of the L7 neuron was removed. The L7 neurites were maintained in culture for 24–48 hr, after which the experiments were performed. Sensorimotor cocultures, each comprising a single pleural sensory neuron and a single LFS motor neuron, were made, and experiments were performed on them as described previously [5].

Electrophysiology

The electrophysiological methods used in the experiments on isolated motor neurons and sensorimotor cocultures were identical to those used in earlier studies [5, 7]. In brief, cultures were perfused with a solution consisting of 50% L-15/50% artificial seawater (ASW) during electrophysiological recording, which was performed at room temperature. For experiments on isolated motor neurons, the cell body—or, in the case of the experiments on isolated neurites, the stump of the severed major neurite—was impaled with a sharp microelectrode (20–30 MΩ) and held at ~−85mV (−0.2 to

−0.6 nA holding current) throughout the experiment. Glutamate (2 mM in perfusion medium with 0.02% Fast Green) was pressure ejected from a micropipette onto the initial segment of the motor neuron's major neurite or the proximal stump of the L7 neurite. The duration and pressure of the ejection pulses used to deliver glutamate were adjusted at the start of each experiment so that an initial response of 5mV–30mV in isolated neurites and 5mV–15mV in isolated motor neurons could be evoked; thereafter, the ejection pulses were held constant throughout the experiment. The glutamate was washed out immediately after application via a rapid perfusion system.

For experiments on in vitro sensorimotor synapses, the presence of a chemical synaptic connection was first ascertained by the injection of a brief pulse of positive current (40 ms, 0.2–0.8 nA) into the sensory neuron and the recording of the resulting EPSP, if any. Given that the initial test pulse elicited an EPSP, gelonin was injected into some of the motor neurons via the recording electrode with air pressure (10 ms pulses, 1–5 psi; Pico-Spritzer II, Parker Hannifin [Fairfield, NJ]). The injection solution consisted of 25 μM gelonin, 0.5 M potassium acetate, 10 mM Tris-HCl (pH 7.5), and 0.2% Fast Green so that the injection could be visualized. In additional control experiments, the vehicle solution was pressure injected into the motor neuron. The synapses were then rested for 30–45 min. Afterward, the synapses were stimulated once every 5 min throughout the experiments.

5-HT was prepared fresh daily as a 2 mM stock solution dissolved in ASW. The 5-HT was diluted to a final concentration of 10–20 μM just before an experiment in the perfusion medium and applied to the cultures for 10 min, after which it was rapidly washed out with normal perfusion medium. Cycloheximide was dissolved in 0.1% dimethyl sulfoxide (DMSO). Emetine was simply dissolved in perfusion medium. Emetine or cycloheximide was added to the bath 20 min before the start of testing, unless otherwise indicated. All drugs were from Sigma (St. Louis, MO), except for gelonin (Aczon [Bologna, Italy]).

Statistical Analyses

For the experiments on isolated motor neurons or neurites, the peak amplitudes of the evoked glutamate potentials (Glu-EPs) were normalized to the mean amplitude of the 60 Glu-EPs immediately before the application of 5-HT, 5-HT-drug, or vehicle—or to the amplitude of the Glu-EP on the 0 min trial in the experiments presented in Figures 1C and 1C′—and expressed as the percent mean ± the standard error of the mean (SEM). Parametric tests were used for all statistical analyses. For the experiments involving either the isolated motor neuron or the isolated neurite group, differences were assessed with the 40 min time point. A one-way ANOVA was performed on the data for the 40 min time point, and Student-Newman-Keuls (SNK) post-hoc tests for pairwise comparisons followed. (Because there were only two groups in the experiment presented in Figures 1C and 1C′, the differences at the 40 min time point were assessed with an unpaired t test.) We wished to determine the earliest time point at which the group differences became significant in the data presented in Figures 2D and 4C. Accordingly, a two-way ANOVA, with time as one factor and treatment as the other, was performed on all of the data; one-way ANOVAs were then performed on the data for each of the time points.

Supplemental Data

One figure is available at http://www.current-biology.com/cgi/content/full/17/23/2073/DC1/.

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